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Award Number: DAMD17-97-1-7050

TITLE: Cloning of Novel Oncogenes Involved in Human Breast Cancer

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REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual Summary (1 Jun 00 - 31 May 01)

4. TITLE AND SUBTITLE
Cloning of Novel Oncogenes Involved in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7050

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

While the aberrant overexpression of HER family receptors is known to be involved in breast cancer, the complex nature of the genetic events that trigger the development of breast cancer remain to be identified. We have developed, refined and applied a retrovirus-based gene transfer assay to identify activated oncogenes in breast cancer. Progress during the past year has involved: (1) the use of breast carcinoma cell lines for generation of retrovirus expression libraries; (2) development of better functional screens for activated oncogenes, and (3) evaluation of protocols to improve the rescue of cDNAs from the transformed cell populations. We have generated several retrovirus-based cDNA expression libraries that represent genes expressed in noninvasive (MCF-7 and T47D) and invasive (MDA-MB4682 and BT549) human breast cancer cell lines. These libraries have been introduced into Rat-1 rodent fibroblasts and RIE-1 rat intestinal epithelial cells and transforming activity was then assayed for. Two isolates from our analyses encode the Raf-1 serine/threonine kinase and the fibroblast growth factor receptor 2.

14. SUBJECT TERMS
Oncogenes, gene transfer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Cancer is a multi-step genetic process involving mutation of oncogenes and tumor suppressor genes (1). With a few notable exceptions (e.g., HER2) the genetic determinants which contribute to the development of breast cancer remain unknown. Conventional techniques using genomic DNA for identifying oncogenes in human tumor cells are cumbersome, inefficient and have met with limited success. While these studies identified the importance of the *ras* and *neu* oncogene in human cancers, they also suggested that cancer arises from the involvement of only a handful of oncogenes (2). Moreover, for practical reasons they have relied on the transformation of fibroblast cell lines to identify oncogenes in epithelial cell-derived tumors. Thus, it seems likely that many oncogenes present in epithelial cell-derived breast carcinomas may not produce an obviously transformed phenotype in fibroblast cells. These limitations argue that oncogenes and tumor suppressor genes that contribute to transformation in human breast cancer remain to be detected. The development of retrovirus vector-based cDNA libraries overcomes the key obstacles to expression cloning of oncogenes contributing to the transformation of epithelial cells (3,4). Thus, we propose to generate retroviral cDNA libraries from human breast tumor cells and screen them for DNA sequences which contribute to the transformation of breast epithelial cells. We believe that this approach will lead to the successful identification of new genetic markers for breast cancer and identify novel targets for the rational design of anti-cancer drugs against breast cancer.

Body

This grant was originally a career development award that funded a former Research Assistant Professor in our research group (Dr. Geoffrey J. Clark) that was then transferred to the current PI this past year. Consequently, we modified some of the approaches that were outlined in his original proposal for our studies. First, the original proposal intended to utilize primary breast tumor samples derived from cancer patients. This approach has been very successful in our library screening studies evaluating activated oncogenes in patient-derived acute myelogenous leukemias (5,6). We did collect 8 breast cancer samples, representing either localized, noninvasive tumor tissue or tumor tissue from invasive cancers. However, we found that while we could isolate good quality mRNA for library generation, we found that the amount of transcripts did not allow for generation of a cDNA expression library that represented the complete genetic complexity of the tumor. Therefore, we have switched our approach to utilize human breast carcinoma cell lines. This first necessitated our profiling of a panel of cell lines for activation of various signaling pathways.

The second facet of these studies required modifications in the screening assays. The original proposal described the use of the human MCF-10A breast epithelial cell line as a screen for novel oncogenes. These efforts were met with significant complications. Therefore, we have opted to use two rodent cell lines, Rat-1 and RIE-1, for our current screens. We have also initiated efforts to develop telomerase-immortalized human mammary epithelial cells as an alternative to MCF-10A cells for subsequent screening efforts (7).

The third facet has been the refinement of technical approaches to isolate the transforming genes from the resulting transformed cell populations. This was necessitated by our

limited ability to isolate the sequences from the majority of transformed cell lines that we isolated. A detailed discussion of this and the other two issues raised above is provided below.

A. **Use of breast carcinoma cell lines as a source of mRNA for library generation.** We felt that one of the key strengths of the original proposal was the use of patient-derived breast cancer tissue for generation of the cDNA expression libraries. We did make progress has involved the isolation of 8 different breast cancer tissue samples for RNA isolation. These represent both noninvasive and invasive tumor tissue. Four cDNA libraries were prepared and two were introduced into the pCTV3 retrovirus vector. However, because of the limited amount of material that can be isolated from patient samples, these libraries did not provide a representation of the complete repertoire of genes expressed in the tumor. In other words, the size of breast tumor samples that would allow us to isolate sufficient mRNA for generation of a complete library was not available to us.

To overcome this limitation, we opted to use established human breast carcinoma cell lines for isolation of mRNA. While this was not an optimal choice, we felt that this was a better option than to screen the incomplete libraries generated from patient tumors.

This change in direction also meant that we had to choose appropriate breast lines for our library generation. In order to do this, we first performed a complete profile of a spectrum of breast carcinoma cell lines that we had in our laboratory inventory. While these lines have been characterized extensively in the literature, in light of lab-to-lab variations in these lines that have been in culture for decades, we felt it was important to know the characteristics of our cell lines.

We used western blot analyses to determine estrogen receptor expression levels, expression levels of HER1-4, activation levels of HER1-4, ERK expression and activation levels, Akt expression and activation levels, and finally, Ras expression and activation levels. The activation levels of HER, ERK, and Akt were done using phospho-specific antibodies. The activation levels of Ras were determined using a pull down assay using a GST fusion protein containing the Ras-GTP binding domain of Raf-1. We have included representative data from these analyses.

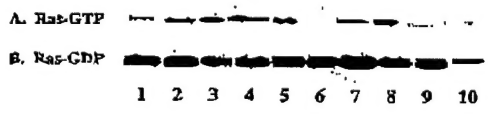

FIG 1: Ras-GTP levels in human breast carcinoma cell lines (A). Lane 1: MCF10A 2: BT549 3: BT474 4: MDA-MB231 5: MDA-MB468 6: T47D 7: MCF7 8: SKBr3 9: Hs578T 10: Zr75. (B) Ras-GDP levels relatively equal in all cell lines. Interestingly, although only

In addition to the analyses of the activity of signaling pathways known to be important in breast cancer growth, we also used three different assays to evaluate the invasive properties of these lines in vitro. These included invasion through Matrigel.

Table 1. Properties of Human Breast Carcinoma Cell Lines

Cell Line	In Vitro Invasion	ERα Protein	Act. HER2	P-ERK	P-Akt	Ras-GTP
MCF-10A	-	+	-	-	-	-
BT549	+	-	-	++	+	+
BT474	-	+	+	++	+	+
MDA-MB231	+	-	-	++	-	+
MDA-MB468	+	-	+	++	+	+
T47D	-	+	-	-	+	-
MCF7	-	+	-	+	+	+
SkBr3	-	-	+	+	-	+
Hs578T	+	-	-	-	+	-
Zr75-1	-	+	+	-	±	-

We have generated several retrovirus-based cDNA expression libraries that represent genes expressed in noninvasive (T47D) or invasive (MDA-MB4682, BT549 and Hs578T) human breast cancer cell lines. The signaling properties of these lines are summarized in Table 1. For example, T47D showed activated Ras and phosphorylated and activated Akt, but no activation of HER2 (or other HERs) or the ERK MAPK cascade. In contrast, both BT549 and MDA-MB468 showed both activated Ras and activated ERKs. Thus, the other activated oncogenes in these two cell lines are likely to be different than those found in T47D cells. By contrast, Hs578T showed no activation of Ras or ERKs, and only limited activation of Akt. Thus, this tumor cell line is likely to express yet another distinct set of oncogenes.

B. **Biological screens for transforming genes** – The original proposal involved the use of the MCF-10A human breast epithelial cell line for the biological screen for transforming genes (8). Unfortunately, we have had two significant problems with the use of this cell line. First, we have found that the cell line tends to "drift" significantly and quickly during routine subculture and passage. The morphology of the cells would change within 4-6 passages and acquire different growth properties. We then acquired additional sources of the cells from other investigators (M. Kinch, Purdue). However, this problem has persisted. Additionally, we found that they could growth without exogenously added EGF and also gave a significant background when suspended in soft agar. The unstable nature of these cells concerned us greatly, since any background transformation in our assays would greatly compromise on our ability to effectively and efficiently isolate transforming genes from our screens.

In order to proceed with our screening without any significant delay, we have chosen to do our current screens using Rat-1 rat fibroblasts and RIE-1 rat intestinal

epithelial cells. Both cell types have proved to be very useful biological screens for activated oncogenes expressed in acute myelogenous leukemias (5,6). While these are clearly not the progenitor cells for breast cancer, they do have several important advantages for these screens. First, unlike the MCF-10A cells, these cells remain stable for prolong periods of subculturing, and their tendency to undergo spontaneous transformation is extremely low. Second, we know that both cell types are response to oncogenes known to cause transformation of human breast epithelial cells. These include the Ras and HER2 oncoproteins. We have also found that they are sensitive to transformation by G protein-coupled receptor and transcription factor oncoproteins. Hence, they can potentially detect a wide spectrum of functionally distinct oncoproteins. Third, we have used both cell lines successfully in our library screens using expression libraries representing genes expressed in patient-derived acute myelogenous leukemias. Finally, our preliminary screens using breast carcinoma cell line-derived libraries have identified multiple transforming genes.

Concurrent with the use of Rat-1 and RIE-1 cells for these screens, we are evaluating immortalized human mammary epithelial cells as possible recipients for our screens (7). Weinberg and colleagues showed recently that normal human primary breast epithelial cells (HMECs) can be immortalized by ectopic expression of the catalytic hTERT subunit of telomerase. The additional expression of SV40 large T and oncogenic Ras was then shown to be sufficient to cause full tumorigenic transformation of these cells. We have acquired these cell lines (C. Counter, Duke) and are evaluating them as recipients for our retrovirus libraries. One issue that we need to address is whether we can convert these cells to be recipients for ecotropic viruses. Since we are concerned that packaging our library in an amphotropic line, to generate virus that can infect human cells, may be a potential biohazard, we want to restrict our analyses and use only virus produced from a ecotropic packaging cell line. Therefore, we need to introduce and express the ecotropic virus receptor to convert these cells to be sensitive to infection with ecotropic virus preps. We have acquired an ecotropic receptor expression vector (R. Khosravi-Far, Harvard) and these studies have been initiated.

One facet of our evaluation of HMECs as recipients for our library screens is to evaluate their sensitivity to tranformation by a variety of oncogenes. Thus, we have taken the HMECs (SV40T + hTERT) and compared their ability to be transformed by activated Ras, with activated Neu, TrkA, Raf, and other oncogenes.

C. **Isolation of transforming sequences** – We have found that the generation of the cDNA expression libraries, when coupled with the very clean transformation assays using Rat-1 or RIE-1 cells, has made it very efficient in detecting transforming activity. Therefore, the isolation of transformed cell populations from our screens has been quite efficient and we have isolated over 50 foci of transformed cells, and we have expanded these populations and have stored each in liquid nitrogen.

The next step, the isolation of the retrovirus-associated transforming gene, has proved to be the rate-limiting step in the entire process. We have struggled with this for many months. The basic aspect of this step is to use oligonucleotide primers that correspond to retrovirus sequences that flank the inserted cDNA sequences in PCR-mediated DNA amplification using total genomic DNA isolated from each transformed cell population. As shown in Fig. 2, our initial attempts met with difficulty in getting clear amplication of sequences, as indicated by a clear fragment when the PCR products were analyzed in agarose gels. This has made it difficult to decide which isolates to pursue for subcloning and further analyses.

We tried initially to improve the quality of the isolated genomic DNA, assuming that this may have been a main limitation of the quality of the PCR-amplified sequences. We tried a variety methods; however, none provides use with a significant improvement in our ability to get clean DNA fragments from our PCR amplifications.

Recently, we have tried different enzymes and conditions for the PCR amplification. The data shown in Fig. 2 compares the PCR products using two different approaches and shows improvements in rescuing the cDNA clone back out from genomic DNA of the 'hits'. The original PCR reaction (for rescuing cDNA) was amplified with Pfu Turbo DNA Polymerase (from Stratagene) using 50 ng of genomic DNA. On the average, our success with PCR-mediated amplification from genomic DNA (only with regards to genomic DNA obtained from the library screens) has been about 1 out of 10 clones. So we've actually had more hits that remain to be identified because we can't always rescue the clones. Recently, we switched to Tth DNA Polymerase (from Clonetech). This enzyme works under reaction conditions that are similar to that for Pfu except with the addition of GC-Melt (a solvent that is used to melt and keep denatured genomic DNA, especially the GC rich regions, remain single strandedlonger to give primers a chance to anneal to the template). Use of this solvent produced single specific amplified products as opposed to multiple bands or sometimes smears. With Pfu, it was unclear what fragment was best to isolate for subcloning and also whether the same sized DNA insert was present in the different isolates. With Tth, it was much more obvious what the insert fragment entailed. After sequence analyses of several isolated sequences, we found that all encode for the fibroblast growth factor receptor 2 (FGFR2).

Key Research Accomplishments

- Profiling of human breast carcinoma cell lines for use as sources of mRNA for the generation of retrovirus cDNA expression libraries – from these analyses we have identified four lines for use in these screens. These include noninvasive and invasive cell lines, as well as those with activated Ras and ERK and without activated Ras and ERK. This will optimize the chances that different oncogenes will be represented in the different libraries.
- Generation of retrovirus cDNA expression libraries representing genes expressed in human breast carcinoma cell lines – in contrast to our libraries made from mRNA derived from patient breast cancer samples, these should be complete and represent the

entire repertoire of genes expressed in each tumor cell line.

- Adaptation and application of Rat-1 and RIE-1 cells lines as biological screens for transforming genes expressed in breast cancer cells – additionally, we are developing and characterizing the HMEC immortalized cells as recipients for our library screens. Ultimately, we feel that these cells may provide the most relevant cell type for our screens.
- Refinement of PCR-based techniques to isolate transforming sequences from transformed cell populations – this technical breakthrough allows us to overcome the rate-limiting step in our library screens. We have plenty of "hits" from the screens, with many transformed cell populations awaiting the molecular cloning of the transforming sequences.
- Identification of Raf-1 and FGFR2 as transforming genes expressed in breast carcinoma cells – as both of these genes are already implicated in growth regulation, these validate the usefulness of the screen for detection of novel transforming activity. To date, we are not aware that either of these full length genes have been identified in library screens. Truncated version of Raf-1 were identified in the early NIH 3T3 screens though.

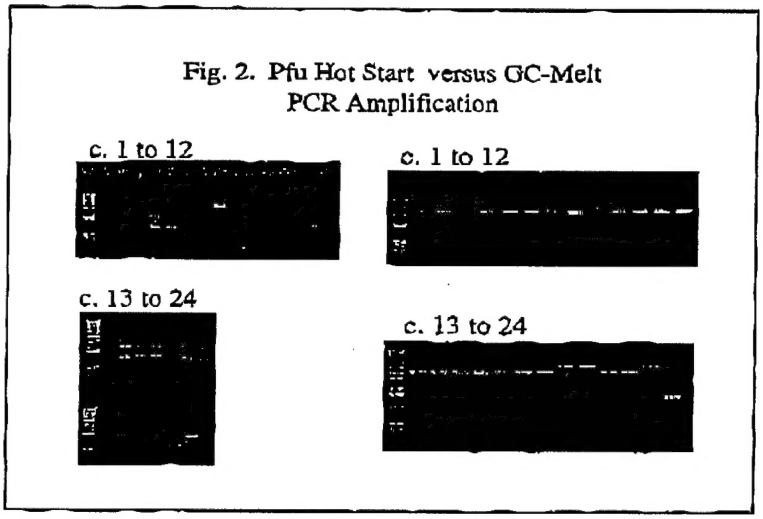

Fig. 2. Pfu Hot Start versus GC-Melt PCR Amplification

Reportable Outcomes

* Abstract and presentation for upcoming Era of Hope Meeting, Orlando, FL 2002

Conclusions

While we are confident that our approach will identify novel oncogenes in human breast cancers, much of our work has concentrated on technology development. The broad goal of these studies was to introduce two major modifications to the previous library oncogene screens. First, the use of primary tumor cell lines directly isolated from the cancer patient. While this has proven to be very effective for our studies of acute myelogenous leukemias, it has been more problematic

for our breast carcinoma library generation. This has involved mainly our inability to obtain sufficiently large tumor samples to allow us to isolate mRNA to generate a complete retrovirus cDNA library. Additionally, the fact that solid tumor samples are quite heterogeneous and includes normal tissue and are vascularized, introduces yet further complexities to this process. For obvious reasons, this has not been a problem for our leukemia library studies.

The second major innovation that we have tried in these studies is the use of human breast epithelial cells for our biological assay. This too has been problematic, since our chosen line (MCF-10A) has not been stable in our hands. This problem has persisted with multiple sources of these cells. Therefore, we have resorted to using two cell lines, Rat-1 and RIE-1, for our current screens. While they clearly are not the progenitor cells for breast cancer, they do provide very sensitive screens with essentially zero background activity. At least these features of the screen are ideal and provide a major improvement over the previous screening efforts using NIH 3T3 cells. Our ultimate goal will be to return to the use of human breast epithelial cells. To achieve this, we are currently evaluating HMECs as possible recipients for our library screens.

A third facet of our studies has been the evaluation of different approaches to isolate the transforming genes from the transformed populations that arise from our screens. In this regard, we feel that we have made an important breakthrough with the identification of a PCR protocol that can efficiently amplify the retrovirus-associated cDNA sequences from total genomic DNA isolated from each transformed cell population. The isolation and analyses of transforming sequences is the rate-limiting step in these studies. Even with our refinements, it still is, but much less so than before. Hence, our ability to isolate multiple transforming sequences will allow us to proceed at a more rapid pace to identify novel oncogenes.

In conclusion, much of our efforts has turned out to be further refinement of the retrovirus library screening assay. We feel that these are important and will allow us to make important new strides in our breast cancer screens. This has been validated in our screens of libraries made from patient-derived leukemia cells, with the identification of multiple novel oncogenes not previously linked to leukemia development, and we hope to extend this success in our breast screens.

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Appendices - None